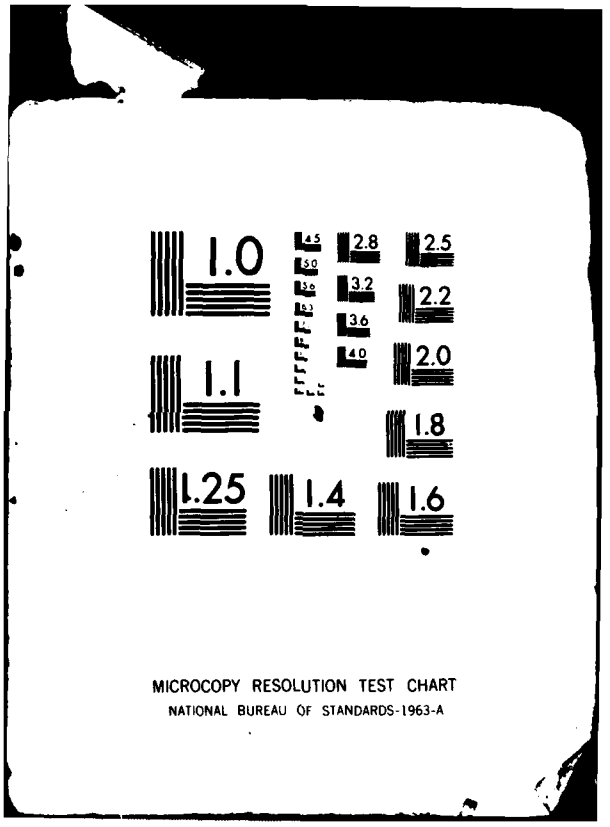
AD-A113 551 DELAWARE UNIV NEWARK COLL OF MARINE STUDIES F/G 6/1
IDENTIFICATION OF MOLECULAR BINDING SITES FOR CALCIUM IN THE OR--ETC(U)
MAR 82 M R CARRIKER N00014-78-C-0045
UNCLASSIFIED CMS-01-82 NL

END
DATE FILMED
4-82
DTIC


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

(12)

AD A113551

Identification of Molecular Binding Sites for Calcium in the Organic Matrix of Molluscan Shell

MELBOURNE R. CARRIKER

CMS-01-82

MARCH 1982

College of Marine Studies • University of Delaware

IDENTIFICATION OF MOLECULAR BINDING SITES FOR CALCIUM IN THE ORGANIC MATRIX OF MOLLUSCAN SHELL

by
Melbourne R. Carriker

A contract report prepared for

Office of Naval Research
Contract N00014-78-C-0045

CMS-02-82

MARCH 1982

Retention file.

College of Marine Studies
University of Delaware
Newark, Delaware 19711

A

INTRODUCTION

The original impetus for this investigation came from observations that the insoluble matrix of calcified tissues from both vertebrates (Glimcher et al. 1965, Glincher and Krane 1968, Davis and Walker 1972) and invertebrates, (Watabe & Wilber 1960), could, when isolated from their parent tissues, influence the rate and form of mineralization from inorganic solutions. Accordingly, we began our research by working exclusively with the insoluble portions of the organic matrix from various mollusc shells. We knew from earlier work that these matrices were largely proteinaceous (Degens, Spencer and Parker 1967) and that they were ubiquitous in the shells of marine molluscs.

We constructed a testable hypothesis from the following seemingly verified information: 1) The insoluble matrix from mollusc shells can be characterized by the part of the shell from which it was removed (Gordon and Carriker 1978); 2) when placed in a solution containing calcium and bicarbonate ions, matrix removed from a calcitic area would, more often than not, cause the growth of calcitic crystals from the solution, while "aragonitic matrix" favored the growth of aragonite (Watabe and Wilbur 1960); 3) when inserted beneath the mantle of a living bivalve, aragonitic matrix would cause growth of aragonite in either part of the animal's shell (Watabe and Wilbur 1960); 4) in the earliest stages of shell deposition, organic matrix could be observed to precede mineralization (Bevelander and Nakahara 1969); 5) the ability of vertebrate organic matrix (bone collagen) to catalyze mineralization was strongly inhibited when carboxyl groups in the protein matrix were modified (Davis and Walker 1972).

The hypothesis can be simply stated: mineralization in molluscs is initiated when charged groups along the protein backbone of shell matrix attract calcium, and perhaps carbonate, ions from solution. The test was also simply devised: Modification or destruction of the responsible charged groups should cause a significant reduction in the removal of calcium ions from solution in the presence of the modified matrix.

Our initial experimental plan, therefore, was to 1) isolate insoluble organic matrix from the shells of various marine molluscs, 2) quantify their ability to remove calcium ions from inorganic solutions of calcium and bicarbonate, similar in concentration to that found in molluscan extrapallial fluid, (Crenshaw 1972a), 3) selectively modify, by chemical means, individual charged moieties on the protein backbone of the matrix, and 4) measure the ability of the modified matrix to alter calcium concentration in solution as per step 2). If, as we expected, one or more such modifications inhibited the rate of removal of calcium from solution, one could infer that at some stage in the mineralization process the binding of calcium to the charged group of interest occurred.

METHODS - I

Living specimens of two bivalves, Mercenaria mercenaria and Crassostrea virginica, and two gastropods, Busycon canaliculatium and Crepidula fornicata, were collected in Delaware Bay in the vicinity of Lewes, Delaware, September to November 1977. Animals were killed by placing them directly into a freezer at -15^{0}C. After excision of soft tissues, shells were scrubbed with a wire brush to remove adherent muscle, mantle and periostracum. Piecas approximately 2 cm on a side were cut from each shell with a rotary diamond saw, and these pieces were

placed in dialysis bags which were subsequently suspended in solutions containing 150 g of sodium ethylenediaminetetraacetic acid (EDTA), with .001 N sodium azide included to inhibit bacterial degradation. pH of decalcifying solutions was maintained roughly between 6.0 and 8.0 by additions, first of sodium hydroxide solution, and later, as the pH began to rise with dissolution of shell, of hydrochloric acid. When no calcium carbonate could be observed within dialysis bags, the pieces were removed and examined for crystalline material under 12X magnification. If no crystals could be microscopically detected, the matrix pieces were returned to decalcification solution for a subsequent 7 days. Depending upon the size of individual pieces of shell, the entire process of decalcification required from 6 to 8 weeks for completion.

At this point dialysis bags were removed from EDTA solution and placed in deionized water. 3 changes of deionized water, at 24 hr intervals, quantitatively removed EDTA from matrix pieces. Some of these pieces were dehydrated in dioxane (Thompson 1966), imbedded in paraffin and sectioned to a thickness of about 8 micrometers with a conventional rotary microtome.

Other pieces of decalcified matrix were incubated in solution made by dissolving calcium chloride and sodium bicarbonate in deionized water. Concentration of the incubation medium was adjusted so that calcium concentration was either equal to or double that found in molluscan extrapallial fluid (Crenshaw 1972a), and the ion product of (Ca^{2+}) (CO_3^{-}) was at least double *in vivo* conditions. For example, a typical incubation solution was made with a final concentration of 9.6 mM Ca^{2+} and 5.0 mM HCO_3^{-}. After the pH was adjusted to 7.64 with Tris-HCl

buffer, the final ion product was 2.19×10^{-6}. Matrix pieces weighing 100 mg $\pm$ 20% (wet wt) were incubated for up to 21 days in 50 ml flasks, tightly stoppered to exclude air. 0.5 ml aliquots were withdrawn at intervals, and calcium concentration measured by titration with EGTA (Tsunogai et al. 1968).

Paraffin-embedded thin sections were dewaxed and brought to water via xylene and ethanol, then incubated for 1 hr at 37°C in 0.1 M $CaCO_3$ solution maintained at pH 7.8 with Veronal buffer. Adjacent control sections were incubated only in veronal buffer. Morin stain, a calcium- specific fluorescent dye (Pearse 1961) was used to detect calcium uptake by thin sections of matrix. Experimental slides were compared with controls under fluorescence microscopy.

RESULTS - I

Decalcified organic shell matrix did not take up calcium from calcium carbonate solution or promote the crystallization of calcium carbonate, after 21 days incubation. In one experiment in which the ion product of calcium and carbonate exceeded saturation, some precipitation occurred and after 30 days calcium concentration dropped from 9.1 mM to 5.4 mM. However, during the same period the calcium concentration in a control flask (containing no organic matrix) decreased from 9.1 mM to 4.3 mM.

No significant fluorescence could be attributed to thin matrix sections which were incubated with calcium carbonate solutions.

INTRODUCTION - II

At this point, near the end of the first year's work, we had come to a fairly important conclusion: that in spite of the evidence published

by Wilbur and Watabe (1961, Watabe and Wilbur 1960), there seemed to be no calcium-mediating function in the insoluble matrix from mollusc shells. There were a few scattered reports in the literature, however, supplying empirical (Crenshaw 1972b, Krampitz et al, 1976) and theoretical (Degens 1976, Weiner and Hood 1975) justification for the belief that soluble protein of mollusc shells is influential in calcium deposition. There was some controversy, though, one investigator claiming that calcium ions are bound by sulfate radicals in the polysaccharide portion of the matrix (Crenshaw 1972B), while a second group (Weiner and Hood 1975) proposed that the molecular spacing and sequence of dicarboxylic acids formed the basis for a functional calcium-binding potential.

We therefore decided to continue the investigation, concentrating on the soluble proteinaceous material of the matrix, but applying the same tests (with minor modifications) we had devised for insoluble material.

METHODS - II.

Shells from the four molluscs mentioned earlier plus two other species Urosalpinx cinerea (gastropod) and Mytilus edulis (bivalve), were decalcified in EDTA solutions prepared as before. Shells were pulverized in a mortar to increase reaction rate with EDTA, and dialysis bags were not used. Decalcification was allowed to continue until no mineral was evident in suspension, typically after one week, Solid materials, i.e. insoluble matrix fragments, were removed by filtration through paper followed by millipore filters. EDTA and calcium salts were removed by ultra-filtration with Amicon PM-10 pressure dialysis membranes; supernatant materials were washed on the membrane with 50 volumes of 0.05 Tris buffer, pH = 7.8 (with sodium azide added), after being concentrated

to about 50 ml. Final separation of matrix proteins from impurities was carried out on Sephadex G-25 gel filtration columns. Typically, 3 ml aliquots of partially purified matrix solution was loaded onto the column and eluted at 50 - 75 ml/hr with 0.05 N Tris buffer, pH = 7.8. 2 ml fractions were automatically collected and protein concentration in each fraction was monitored during elution by ultraviolet absorbance measurement at 280 nm.

Fractions containing detectable protein were assayed for calcium binding ability with a radiotracer method. A fraction was incubated for 30 min. at room temperature with a 0.1 M $CACl_2$ solution containing approximately 0.5 microcuries of ^{45}Ca. The solution was then chromatographed as before on Sephadex G-25. 0.5 ml of each eluted fraction was dissolved in aqueous counting solvent and measured for radioactivity with a Beckman LS-100 liquid scintillation counter. The remainder of each fraction was assayed for total protein by the Coomassie Brilliant Blue method (Bradford 1976).

Aliquots of fractions eluting in the same volume as those determined, in these preliminary experiments, to have calcium-binding ability were subjected to chemical modification procedures designed to attack individual amino acid (or other) residues in the soluble matrix.

1) Conversion of dicarboxylic acids and other carboxyl groups to amides (Hoare and Koshland 1967). By incubating with a solution of 1-ethyl-3-dimethylaminopropylcarbodiimide, carboxyl groups are converted to acylisourea derivatives.

We subsequently reacted the derivatives with 1 M NH_4Cl to form amides, reversing or neutralizing the charge present at the original acidic site.

2) Destruction of both carboxyl and ester sulfate groups by methylation (Thompson 1966). A fraction containing calcium-binding matrix was evaporated under vacuum to a small volume, then redissolved in 1 ml H_2O. Protein was precipitated with 2.5 volumes of ethanol, the precipitate was washed with methanol and incubated for 72 hr at 4^oC with methanol containing 0.1% concentrated HCl. Precipitates were then allowed to air dry after an ethanol wash, and dissolved in 2 ml of distilled H_2O.

3) Dinitrophenylation of proteins with DNFB. Soluble matrix solution was concentrated to 1 ml containing 1.5 mg protein of a PM-10 membrane, and added to 0.5 g $NaHCO_3$ dissolved in 5 ml water. DNFB in ethanol was added, and the mixture stirred for 2 hrs at room temperature, centrifuged, decanted and precipitated with ethanol. Solids were dissolved in water and reconcentrated on the ultrafiltration membrane.

4) Modification of guanidino groups with cyclohexanedione (Means and Feeney 1971). 3 ml of soluble matrix solution was combined with 5 mg of 1,2-cyclohexanedione in 2 ml water. pH of the mixture was adjusted to 11.0 with triethylamine and the contents were stirred in darkness for 24 hrs. Reagents were washed out with 150 ml of 0.05 N Tris buffer on a PM-10 membrane.

5) Modification of phenolic groups with tetranitromethane (Glazer et al. 1975). 2 - 3 ml of sample were adjusted to pH = 8.0 with 0.2 N Tris buffer, and 1 ml of tetranitromethane stock (0.835 M in 95% ethanol) was added. The mixture was stirred for 1 hr, then washed with 300 ml pf 0.05 N Tris buffer on a PM-10 ultrafiltration membrane.

6) Acetylation of amino groups (Glazer et al. 1975). 2 ml of sample were mixed with 2 ml of saturated sodium acetate solution. 20 microliters of acetic anhydride were added, and the mixture stirred for 1 hr over an ice bath. pH was monitored and maintained at 7.8. Reactant mixtures were washed as before with 0.05 N Tris buffer.

Each modified sample was subsequently incubated with radioactive calcium chloride solution; an untreated control aliquot was incubated at the same time under identical conditions.

RESULTS - II

The results of the preceding experiments can be summarized simply: none of the modification procedures was used had a substantial effect on the calcium binding capacity of the matrix samples tested. Calcium binding varied between about 1.0 and 3.0 x 10^{-9} mols calcium per microgram of protein, as assayed by the Coomassie Blue method. Inter-specific differences in calcium binding capacity cannot be evaluated because molecular weight determinations have not been performed on the various matrix proteins that were tested. It should also be noted that procedures used eliminated those compounds present in the matrix of the shells that were dialyzable by Amicon Pm-10 membranes.

The major finding of this part of the work is typified by Figures 1 and 2. In Figure 1, calcium binding is seen in an untreated aliquot of matrix from the shell of Mercenaria mercenaria, at the top of the figure. A peak of radioactivity resides beneath the peak of ultraviolet absorbance in the void volume of the Sephadex column. The lower half of the figure shows absorbance and radioactive traces for an aliquot of the same material which has been modified by treatment with acidified methanol.

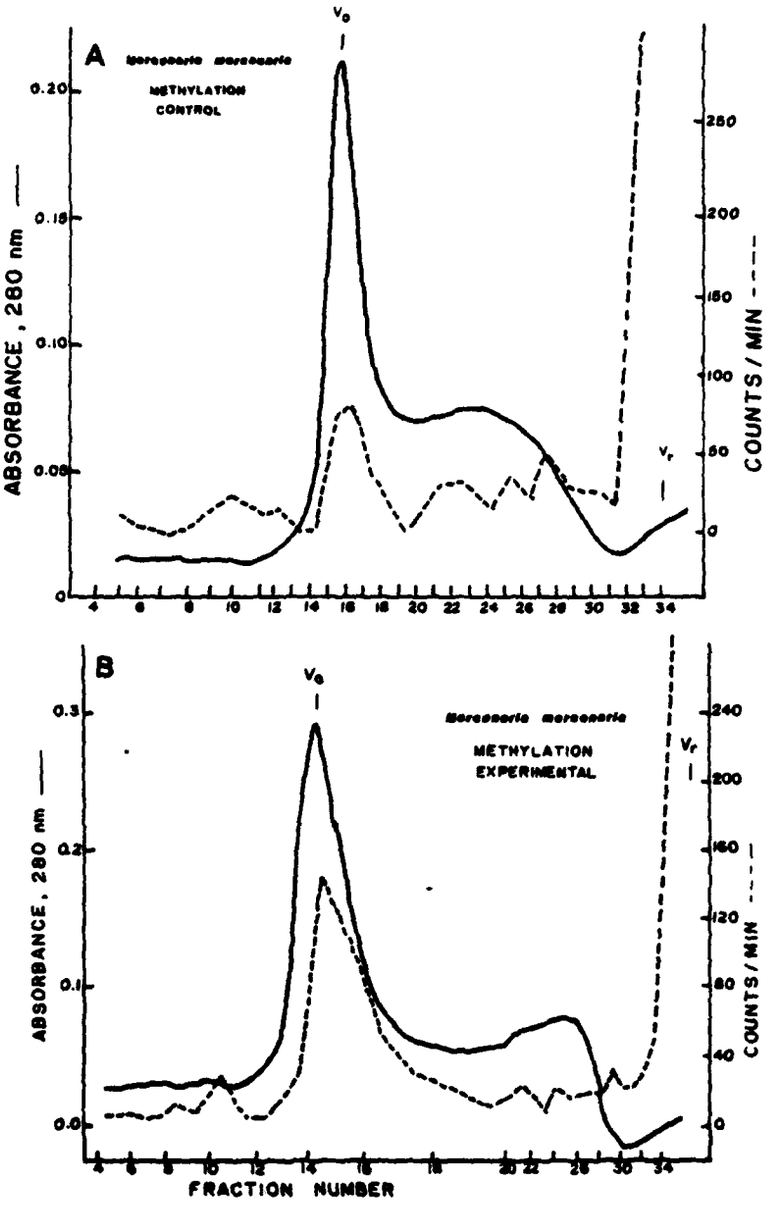


Figure 1. Calcium binding in the soluble matrix from the shell of the bivalve Mercenaria mercenaria. a) Ultraviolet absorbance (solid line) plotted on the same axes as radioactivity per fraction (dashed line), in fractions of untreated soluble matrix. Fractions eluted from a Sephadex G-25 column with 0.05 N Tris buffer. b) Ultraviolet absorbance and radioactivity in fractions of soluble matrix methylated with acidified methanol.

In this case, destruction of sulfate groups has not diminished calcium binding.

Figure 2 illustrates a similar sequence for the shell matrix of the whelk Busycon canaliculatum. In this case the treatment involved modification of dicarboxylic amino acids by reaction with ammonium chloride after activiation of the protein with the carbodiimide EDC (procedure 1. described in methods section). Reversing the charges present on glutamic and aspartic acid residues did not reduce the calcium binding ability of the protein.

Similar results were obtained for all other chemical modification procedures tested in this program.

METHODS - III

To investigate the origin of calcium-binding proteins in the matrix of the shell, we used a radioactive assay on living animals, modifying a procedure first published by Veitch (1974).

Live quahogs, Mercenaria mercenaria, approximately 7 cm in length, were taken from sand flats at low tide in Lewes harbor, Delaware. An area central to one valve was scrubbed and washed with methanol. A threaded plexiglas fitting was cemented in place over the cleaned area with fast-setting epoxy. In the center of the fitting a 2 mm hole was drilled through the shell into the extrapallial space with a diamond core drill. After flushing with water to remove drilling chips, the fitting was closed off by screwing a plexiglas cap, containing the O-ring seal, in place (Figure 3). Drilled animals were kept in a 25 gallon aquarium and fed regularly with the diatom Thalassiosira pseudonanna.

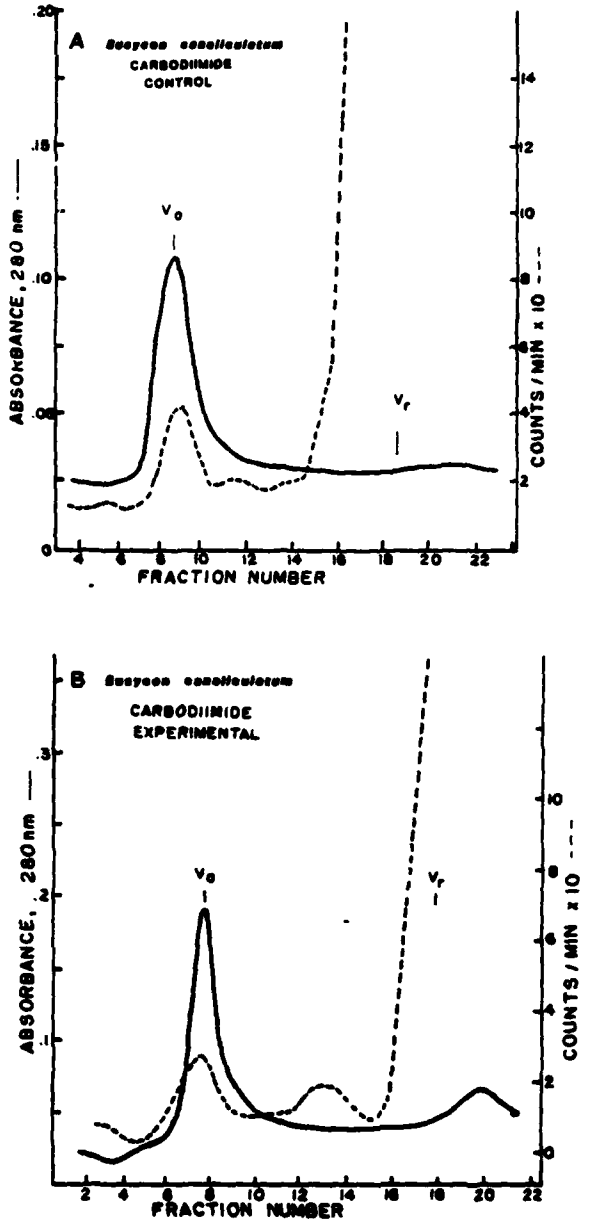


Figure 2. Calcium binding in the soluble matrix from the shell of the gastropod Busycon canaliculatum: a) Ultraviolet absorbance (solid line) plotted on the same axes as radioactivity per fraction (dashed line), in fractions of untreated soluble matrix. Fractions eluted from a Sephadex G-25 column with 0.05 N Tris buffer. b) Ultraviolet absorbance and radioactivity in fractions of soluble matrix treated with carbodiimide and ammonioum chloride to neutralize dicarboxylic acids.

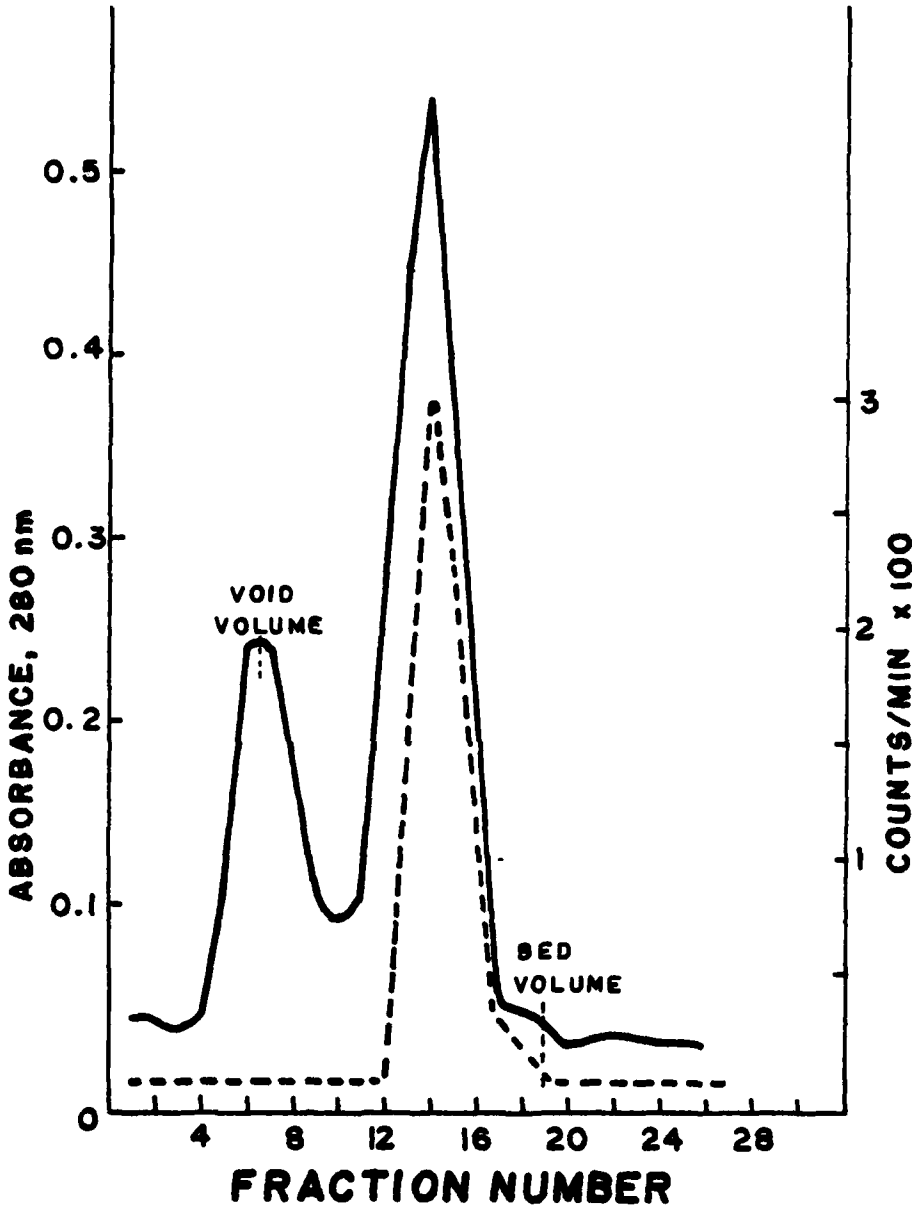


Figure 3. Exclusion chromatography of extrapallial fluid extracted from Mercenaria mercenaria which was incubated with radioactive Ca-45. Absorbance a 280 nm plotted against number of eluted fractions, 3.5 ml per fraction, solid line. Radioactivity in counts per minute from liquid scintillation counter plotted against fraction number, dotted line. Sephadex G-25 gel, eluted with 0.05 N Tris buffer, pH = 8.0.

Amino Acid	Residues per 1000
ASP	99
THR	47
SER	59
GLU	44
PRO	13
GLY	73
ALA	101
ILU	34
LEU	75
HIS	417
LYS	37

Table 1. Amino acid analysis of calcium-binding fraction of *Mercenaria mercenaria* extrapallial fluid (eluted from sephadex G-25 column with 0.05 N Tris buffer, pH = 8.0).

Experimental animals were removed to individual 8 liter aquaria where they were allowed to pump and feed for 24 hours in sea water to which 50 microcuries of radioactive calcium chloride had been added. At the end of the experimental period the clams were removed from the water and washed thoroughly with tap water followed by distilled water. The O-ring cap was then removed, and a sample of extrapallial fluid was removed with a syringe. After centrifugation to remove particulates, the extrapallial fluid samples were dissolved in an equal volume of 0.050 Tris buffer and applied to a column of Sephadex G-25. The fractions were monitored as before for UV absorption and radioactivity.

Fractions showing highest radioactivity were submitted for amino acid analysis.

RESULTS - III

Figure 3 shows that all of the bound radioactivity in the sample of extrapallial fluid is found in the included volume. The position of the radioactive peak indicates that the molecular weight of the calcium-binding molecule is close to 2500 daltons. No radioactivity appears in the void volume.

Amino acid analysis showed that the compound was a protein containing a suprisingly high histidine concentration (Table 1). Alanine and aspartic acid were next highest in content, but not remarkably higher than the remaining amino acids.

CONCLUSIONS

We have found calcium-binding activity, on the order of 1 to 3 x 10^{-9} mols calcium per microgram of matrix protein, in soluble matrix extracted from the shells of a variety of marine mollusks. Modification

or destruction of a number of chemical groups, previously postulated to be active calcium-binding sites, failed to destroy or diminish the uptake of calcium in soluble proteins above 10,000 daltons molecular weight. Previously it had been suggested that mineralization in mollusks was initiated by binding of one or more calcium ions to dicarboxylic acids (Weiner and Hood 1975) or to ester sulfate groups (Crenshaw 1972). These hypotheses are weakened by the evidence found in this study.

Wilbur and Watabe (1963, Watabe and Wilbur 1960) found that the presence of insoluble matrix, both in vitro and in vivo, influenced the growth patterns of shell in bivalves. From this it might be inferred that the insoluble portion of shell matrix is responsible either for modifying the kinetics of calcium carbonate growth from solution or for initiation of crystal growth by binding of calcium. We found no evidence for either phenomenon.

We have developed a technique for simply and repeatable carrying out in vivo assays of bivalve extrapallial fluid under a variety of experimental conditions. Using this technique we showed that calcium in sea water is taken up by the clam Mercenaria mercenaria and bound to a low molecular weight proteinaceous compound in the extrapallial fluid. The calcium-binding protein is very rich in histidine, and its molecular weight is probably considerably lower than reported by Crenshaw (1972).

LITERATURE CITED

Bevelander, G. and H. Nakahara, 1969. An electron microscopic study of the formation of the nacreous layer in the shell of certain bivalve molluscs. Calc. Tiss. Res. 3: 84-92.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72: 248-254.

Crenshaw, M. A., 1972a. The soluble matrix from Mercenaria mercenaria shell. Biomin. Res. Repts. 6: 6-11.

Crenshaw, M. A., 1972b. The inorganic composition of molluscan extrapallial fluid. Biol. Bull. 143: 506-512.

Davis, N. R. and T. E. Walker, 1972. The role of carboxyl groups in collagen calcification. Biochem. Biophys. Res. Comm. 48: 1656-1662.

Degens, E. T., 1976. Molecular mechanisms on carbonate, phosphate and silica deposition in the living cell. Topics Current Chem. 64: 1-112.

Degens, E. T., D. W. Spencer and R. H. Parker, 1967. Paleobiochemistry of molluscan shell proteins, Comp. Biochem. Physiol. 20: 553-579.

Glazer, A. N., R. J. DeLange and D. S. Sigman, 1975. Chemical modification of proteins. New York: American Elsevier Publishing Company.

Glimcher, M. J., A. Andrikides and D. Kossiva, 1965. The alteration of amino acid side chain groups of collagen and its effect on in vitro calcification. In: The Structure and Function of Connective and Skeletal Tissues, ed. by G. R. Tristram and S. Fitton-Jackson. London: Butterworths, p. 342-347.

Glimcher, M. J. and S. M. Krane, 1968. The organization and structure of bone, and the mechanism of calcification. In: Treatise on Collagen, ed. by B. S. Gould. New York: Academic Press. p. 68-241.

Gordon, J. and M. R. Carriker, 1978. Growth lines in a bivalve mollusk: subdaily patterns and dissolution of the shell. Science 202: 519-521.

Hoare, D. G. and D. E. Koshland, 1967. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. J. Biol. Chem. 242: 2447-2453.

Krampits, G., J. Engels and C. Cazaux, 1976. Biochemical studies on water-soluble proteins and related components of gastropod shells. In: The Mechanisms of Mineralization in Invertebrates and Plants, ed. by N. Watabe and K. M. Wilbur. University of South Carolina Press.

Means, G. E. and R. E. Feeney, 1971. Chemical Modification of Proteins. San Francisco: Holden-Day Inc.

Pearse, A. G. E., 1961. Histochemistry Theoretical and Applied, Boston: Little Brown and Company.

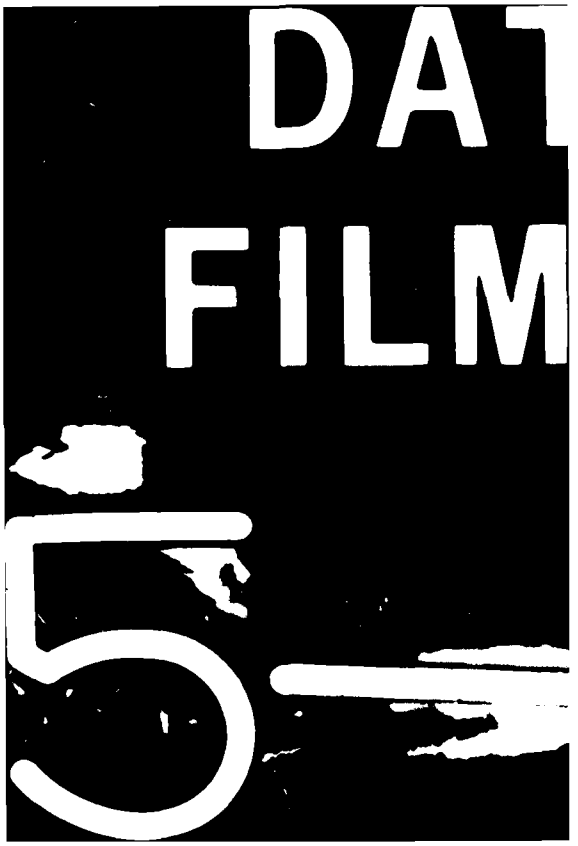
Thompson, S. W., 1966. Selected Histochemical and Histopathological Techniques. Springfield, IL: Charles C. Thomas.

Tsunogai, S., M. Nishimura and S. Nakaya, 1968. Complexometric titration of calcium in the presence of large amounts of magnesium. Talanta 15: 385-390.

Watabe, N. and K. M. Wilbur, 1960. Influence of the organic matrix on crystal type in molluscs. Nature 188: 334.

Weiner, S. and L. Hood, 1975. Soluble protein of the organic matrix of mollusk shells: a potential template for shell formation. Science 190: 987-989.

Wilbur, K. M. and N. Watabe, 1963. Experimental studies on calcification in molluscs and the alga Coccolithus huxleyi. Ann. N. Y. Acad. Sci. 109: 82-112.


DAT
FILM